Development of a Novel In Vitro Co-Culture System for Discrete Subaortic Stenosis

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Abstract

The aim of this study was to investigate the survival, distribution and reaction of different cell types on a monolayer disk, as well as their behavior under bioreactor treatment. Specifically, porcine EEC and porcine fibroblasts (PCF) were labeled with GFT and Texas Red, respectively, to track their viability and distribution. The experiments involved monitoring the cells using various microscopy techniques and comparing the results with controls. These findings have important implications for understanding cell behavior and potential applications for Discrete Subaortic Stenosis. This paper aims to discuss the implications of the findings in the context of existing literature and future research directions.

Introduction

Children with Discrete Sub-Aortic Stenosis (DSS) experience a narrowing of the left ventricular outflow tract (LVOT), caused by the formation of a fibro membranous tissue. This blockage increases the pressure in the LVOT, which is located just below the aortic valve [1,2]. DSS is an uncommon occurrence in children with congenital heart defects, with a prevalence rate of around 6% [3˜6]. The pathogenesis of DSS is hypothesized to begin during the first 10 years of life [6]. Symptoms include chest pain, heart failure, syncope, with about 50% of patients having a heart murmur that grows with age [5,7]. The morphology of the lesions associated with DSS typically consists of a fibromuscular tissue ring or a localized protrusion of fibrous tissue [8]. It is believed that the condition is initiated by geometric abnormalities in the LVOT, which generates turbulent blood flow and altered shear forces that trigger a fibrotic response [9˜14]. Although surgical resection of the membranes provides relief, there is a high risk of recurrence (20-30%) in what is defined as an aggressive DSS phenotype, with female sex being a risk factor [15˜17]. Without Intervention, DSS can lead to left ventricle hypertrophy, aortic regurgitation, endocarditis, arrhythmias, and in extreme cases, death [18˜22].

Surgical correction of the membrane has been demonstrated to be a viable option for eliminating obstruction. The membrane will either be removed via myomectomy, or just the fibrous tissue ring resected [123]. However, it has the potential to pose numerous risks, such as those associated with anesthesia, sternotomy, and heart bypass [4]. In extreme cases of aggressive DSS, open-heart surgery is a higher risk, thus patients undergo percutaneous balloon dilation [24]. This procedure lasts months to a few years, and then surgery must be performed [5]. These surgical approaches don't last, and most patients will require reoperation, due to regrowth of the membrane, and long-term follow up care [1, 5, 23, 25].

It has been observed that DSS lesions are composed of 5 layers of endothelial and fibroblast cells [26, 27]. Researchers have hypothesized that the increased blood pressure, or turbulent jet due to DSS lesions on the LVOT, narrows the subaortic tract, causing a disturbance in cellular function in these layers [6, 28]. Observations have shown that endothelial cells release growth factors when subjected to shear stress above

normal levels, inducing proliferation of other cells, such as fibroblasts, that comprise the blood vessel layers and influencing the mechanical properties of blood vessels [1, 14, 29, 30, 31]. In a similar but separate study conducted showed that endothelial cells, when exposed to shear stress, induce the release of basic fibroblast growth factor (bFGF) that stimulates the proliferation of fibroblasts [30]. Current studies are still limited in understanding a consistent pattern of pathogenesis of DSS that can be targeted for therapies and diagnostic options for pediatric patients with this disease.

Although 2D cell culture studies allow researchers to understand the biology and morphology of cells and tissues, it is limited in the accuracy of how cells interact with the extracellular environment and different cell types [32˜34]. 3D models allow for a more accurate study by mimicking disease conditions [35, 36]. With 3D models we can reproduce the authentic characteristics of the heart's tissue organization and microenvironment [34˜38]. As previously determined, DSS is hypothesized to be caused by interactions between 2 different cell types, EECs and Fibroblasts [39,40]. Conventional cell culture systems and models are centered around a single cell type, which is vastly different from the natural environment of the cell [41, 42]. Thus, this limitation makes it difficult to understand the mechanistic and biological properties of the disease. Creation of a 3D co-culture system that eliminates the disadvantages of a traditional culture system that is restricted in mimicking the natural environment and interactions between cells and extracellular components [42˜45].

We aim to replicate the functional response of fibroblasts in DSS patients and transition from a monolayer 2D culture to a 3D co-culture system. To gain an understanding of DSS progression, several aspects of the tissue must be considered. First, the tissue is 3D in nature, i.e., the fibroblasts are organized within a complex 3D ECM matrix. Second, the matrix will become stiffer over time, i.e., dynamically changing stiffness. Third, the relative spatial positions of the EECs and the fibroblasts are more complex than can be replicated in simple 2D monolayer cultures. In this article we will show the development and optimization of a 3D co-culture system with porcine cardiac fibroblasts and endocardial endothelial cells and subject the model to shear stress conditions as observed in DSS.

Materials and Methods

Gelatin from bovine skin powder (9000-70-8), Calcium Chloride pellets (10043-52-4), and sodium alginate (9005-38-3) obtained from Sigma Aldrich. Commercial Cellink BIOX Printer (Model: S-10001-001; S/N: 202041). ECM media (Sciencell, 1001), Fibroblast growth media 3 (PromoCell Inc. C-23025). 0.05% Trypsin EDTA (Gibco 25300054).

Research Design

4 different types of disks were fabricated. The 1stgroup contained only EECs in all 3 layers, adding $3x10^5$ EECs per layer. The 2nd group contained only Porcine Fibroblasts in all 3 layers, $3x10^5$ Porcine Fibroblasts per layer. The 3rd group of disks were comprised of 2 layers of Porcine Fibroblasts and 1 layer of EECs as the top layer, with same ratio of cells per layer as the first 2 groups. Lastly, the 4th group of disks contained 3 layers of contained Porcine Fibroblasts and EECs dispersed throughout all 3 layers,$3x10^5$ EECs and $3x10^5$ Porcine Fibroblasts in each layer for a total of $6x10^5$ cells per layer. Cells were cultured in the disk for 48 hours and then each group subjected to shear stress in the cone-and-plate bioreactor at 6 dynes/cm2 for each group for 24 hours. Before and after inducing shear stress, confocal images were collected of each layer of each disk.

Cell culture

Porcine Cardiac Fibroblasts and Endocardial Endothelial Cells were cultured each cell type on an individual monolayer of 0.1% gelatin, seeding $5x10^5$ cells. EECs and Porcine Fibroblasts were maintained in ECM media and Fibroblast Growth Media 3, respectively. Once cells reach confluency and before trypsinization, each cell type was labelled using CellTrackers. To be more specific, the Porcine EECs were labelled by GFP (ThermoFisher, Cat #Q25041MP), and Porcine Cardiac Fibroblasts were labelled by Texas Red (ThermoFisher, Cat # Q25021MP). We then detached them using 0.05% Trypsin EDTA. We then cultured the Celltracker-labelled cells as previously mentioned.

Optimization of Bioink with Cells –

In previous studies [35], we established that 8% alginate produced the best results of reproducibility, ease of crosslinking, and appropriate stiffness. We established the appropriate seeding density of cells in alginate hydrogel by using a 3-way luer lock to combine one syringe containing 1mL of 8% alginate with a separate syringe containing 200uL of 1x10ˆ6 cell suspension. After printing the disks, confocal images were collected and analyzed to determine that 1 million cells in 200uL of media is optimal cell concentration to print the disks. Then, printed under sterile conditions using cellink.

Disk Fabrication

From the Fig 1., the disks were developed through a 4-step process. Firstly, we created an STL file using bioprinter that formulates a set of instructions on how to build a disk with 3 dimensions through programmed printhead movements in the x, y, and z planes [35]. The disks were 55 mm in diameter. The file was then transferred to the 3D printer, where it is sliced into layers and printed as a grid pattern. In order to ensure the disk can withstand the cone-and-plate bioreactor, we utilized the printers' customizable features to modify the number of layers of the disk, inserting 2 additional layers, 0.1mm each.

3g of gelatin was combined with 30mL of 0.2% CaCl2, sodium alginate crosslinking agent. After 10 minutes, the gelatin mixture was centrifuged and plated to fill the entire volume of the Nunclon dish. Next, the cartidridge containing 1mL of alginate with 200uL of cell suspension is attached to a stainless steel dispense tip (Nordson EFD, Cat #7018345), which is calibrated to a position 10mm deep into the gelatin support bath. Once printing is complete, the dish is placed in the incubator for 2 hours, to allow the gelatin to completely melt. Lastly, the gelatin is aspirated and 20mL of media with 1% CaCl2 is added to supply nutrients to the cells imbedded in the disk and allow the disk to achieve maximum stiffness over 24 hours. The disk is then stored in the incubator.

Application of Shear Forces

This study uses the cone and plate bioreactors (CPB) to investigate the fluid shear stress experienced by endothelial cells (EECs) in the left ventricular outflow tract (LVOT) of patients with DSS. The CPB system operates based on the following principle: an inverted cone is positioned above a stationary flat plate, creating a narrow gap through which fluid flows. This arrangement ensures the distribution of uniform shear stress across the surface [46].

To control the fluid flow, a magnetic stir plate is employed, which enables adjustment of the cone's angular velocity by modifying the revolutions per minute (RPM) setting on the control unit. This mechanism allows for precise manipulation of the shear stress experienced by the EECs in the CPB system [46].

Fixing the Disks and Confocal Microscopy imaging

The Disks containing cells were initially cut to half or small pieces and transferred onto a 6-well plate and then fixed using a 4% paraformaldehyde solution for 24 hours. Following the fixation, the paraformaldehyde solution was removed, and the disks were washed three times with PBS. The Disks were subsequently imaged and processed using a CellVoyager CV8000 Yokogawa confocal scanner microscope. For imaging, a z-depth range of -150µm to 550µm was selected, with a scanner layer thickness of 6.5µm. The lasers used were 640nm for PCF labeled with Texas Red and 488nm for EEC labeled with GFP. The objective lens chosen for observing the Purkinjean was the 4x lens. The acquired images were then merged and analyzed using CellPathfinder (a high-content analysis software developed by Yokogawa) to generate 2D or 3D aligned images.

Results

The Cells are alive on the Monolayer Disks with the labelling system.

To detect the cells whether or not survive after using celltrackers, as mentioned above again, the Porcine EEC were labeled with GFT and PCF were labeled with Texas Red. From the Fig. 2, the cells are observed

by Lecia microscope with 10x and 20x lens after 72 hours. Thus, different types of the cells are alive after using celltrackers. Then, the cells are transferred to the Monolayers (Disks), using the ECM medium for the Porcine EEC and Porcine Fibroblasts were maintained in Fibroblast Growth Media 3. After culturing for 72 hours, the Monolayers were fixed and the images were taken by Yokogawa CellVoyager CV8000 under the 4x objective in the Fig 3. The images were from different z-depth such as -50, 100, 350 and 500 μm, and cells are widely distributed in the Monolayers.

The mixed cells survived on the multiple layers

To investigate that whether or not the PCF and Porcine EEC survive in the multiple layers (Disks), we mixed the cells and transferred them to the multiplex layers. After fixing and washing the Disks, we still utilized the Yokogawa CellVoyager CV8000 to monitor the cells. From the Fig 4, we found that the Porcine fibroblast- Texas Red and EEC- GFP were mixed and survived after 48 hours in the Disks. Also, from the images, the cell numbers of PCF and Porcine EEC were different at the different z-depth of 100 and 200 μm. From the images, we confirmed that the PCF and Porcine EECs were alive after bioprinting and mixed together with conditioning mediums.

The mixed cells on the Disks were alive after bioreactor treatment.

To further detect the mixed cells on the Disks whether can be potential facilitated for DSS project, we utilized the cone and plate bioreactors since it can imitate the heart fluids as Fig.1 & Fig. 6 showed the schematic and model diagrams. In addition, we designed two groups to conducted for experimental comparing, one group is control group without bioreactor treatment, and the other one is the experimental group within bioreactor treatment for 24 hours. During the bioreactor treatment, we also used the 1:1 ratio conditioning medium as mentioned above. In addition, the Disks were all fixed by 4% PFA and sectioned for Yokogawa CellVoyager CV8000 to observe the cells. From the Fig 5, the Disks under bioreactor treatment seems to be widely spread or distributed comparing to the 0-hour control group. Also, Disks after bioreactor treatment had less cells comparing to control groups, especially the PCF labeled with Texas-Red. Additionally, cells are still alive and stayed on the Disks after bioreactor treatment.

Discussion:

Preliminary experiments showed that labeled Porcine EEC and PCF cells remained viable after being labeled with a cell tracker and transferred to a single-layer disk. Observation of living cells with a Leica microscope confirmed the successful labeling and survival of different cell types. In addition, images of Yokogawa CellVoyager CV8000 at different z depths show that the cells are widely distributed within a single-layer disk, indicating their ability to adhere and proliferate on the disk surface. This finding is consistent with previous studies that have shown that monolayer cultures are suitable for supporting cell growth and maintaining viability [47].

The survival of mixed PCF and porcine EEC cells in multilayer disks was investigated with encouraging results. Yokogawa CellVoyager CV8000 images showed that Porcine fibroblasts - Texas Red and EEC-GFP cells co-existed and were viable inside the disks after 48 hours. Importantly, the different cell numbers observed at different Z-depths indicate a pattern of differential potential distribution within the multilayer. This finding is consistent with research highlighting the importance of spatial organization and cell-cell interactions in mixed cell cultures [48]. Future research should focus on characterizing the spatial arrangement and intercellular communication between different cell types within the composite layer.

The simulation of cardiac fluid using cone and plate bioreactors has provided valuable insights into the mixed cell populations under conditioning medium. There were significant differences in cell distribution between the control group and the experimental group after 24 hours of bioreactor treatment. Yokogawa CellVoyager CV8000 images showed that disks exposed to bioreactor processing showed a wider distribution of cells compared to controls. This observation suggests that the dynamic fluid environment promotes the dispersion of cells within the disk. In addition, a reduction in the number of cells in the petri dish after bioreactor treatment, particularly PCF labeled with Texas red, may indicate potential cell detachment

or altered cell survival ratio under the shear stress conditions. Future research could explore the specific mechanisms of cell detachment under bioreactor conditions and potential differences in cell viability and function.

In this study, the bioreactor provided the shear stress was used as a model to study the DSS. The present study investigated the co-cultural system under bioreactor treatment. The results showed that the mixed cell population successfully survived, distributed, and reacted on a monolayer disk, highlighting the behavior of these cells under bioreactor treatment. The findings of this study provide valuable insights into cell behavior in DSS.

The use of bioreactors in co-culture systems can significantly affect cell distribution from the results. The decrease in the number of cells observed in the bioreactor treated samples suggests that shear stress caused by the flow dynamics of the bioreactor may lead to cell detachment. This finding is consistent with previous research and highlights the importance of optimizing bioreactor conditions to maintain cell adhesion and prevent cell loss during culture. Understanding the effects of shear stress on cell behavior is critical to successfully implementing a co-culture system in DSS.

Also, the successful survival and distribution of a mixed cell population in a co-culture system including single layer and multiple layers, which demonstrates the potential for a more holistic approach in DSS applications. By combining multiple cell types, such as endothelial cells, and fibroblasts, it is possible to develop more efficient tissue structures for DSS repair or replacement [49].

The co-culture system is also designed to address the limitations of single-cell type therapy. By integrating multiple cell types that are critical to the formation and function of target tissues, researchers can create tissue structures that more closely resemble natural tissues [50]. For instance, the addition of endothelial cells helps promote blood vessel formation, smooth muscle cells support mechanical properties, and fibroblasts contribute to extracellular matrix production and tissue stability in DSS [51].

By providing a more comprehensive and versatile approach, co-culture systems have the potential to create organizational structures that better mimic the complexity and function of native tissues [52]. This approach allows researchers to study cell-cell interactions, signaling pathways, and tissue development in a more physiologically relevant way. Ultimately, it provides more efficient and functional tissue structures to improve the treatment for DSS patients.

Conclusion:

The results of this study demonstrate the successful survival, distribution, and reaction of mixed cell populations on a monolayer disk, as well as their behavior under bioreactor treatment. The results showed the viability of the labeled cells and their ability to proliferate in single - and multilayer (Disks) cultures. The use of bioreactors affects cell distribution and results in a decrease in cell numbers, which may be due to detachment caused by shear stress. These findings contribute to the understanding of cell behavior in engineered systems and have implications for DSS applications.

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